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**Title: EARLY EXPRESSION OF THE FRACTALKINE RECEPTOR CX3CR1
IN PANCREATIC CARCINOGENESIS**

Running t.: CX3CR1 EARLY EXPRESSION IN PANCREATIC CANCER

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ABSTRACT

Background. In pancreatic ductal adenocarcinoma (PDAC), fractalkine receptor CX3CR1 contributes to perineural invasion (PNI). We investigated whether CX3CR1 expression occurs early in PDAC and correlates with tumor features other than PNI. **Methods.** We studied CX3CR1 and CX3CL1 expression by immunohistochemistry in 104 human PDAC and coexisting Pancreatic Intraepithelial Neoplasia (PanIN), and in PdxCre/LSL-*Kras*^{G12D} mouse model of PDAC. CX3CR1 expression *in vitro* was studied by a spheroid model, and *in vivo* by syngenic mouse graft of tumor cells. **Results.** Fifty-six (53.9%) PDAC expressed CX3CR1, 70 (67.3%) CX3CL1, and 45 (43.3%) both. CX3CR1 expression was independently associated with tumor glandular differentiation ($p=0.005$) and PNI ($p=0.01$). PanINs were more frequently CX3CR1+ (80.3%, $p<0.001$) and CX3CL1+ (86.8%, $p=0.002$) than matched cancers. The survival of PDAC patients was better in those with CX3CR1+ tumor ($p=0.05$). Mouse PanINs, were also CX3CR1⁺ and -CL1⁺. *In vitro*, cytokines significantly increased *CX3CL1* but not *CX3CR1* expression. Differently, CX3CR1 was up-regulated in tumor spheroids, and *in vivo* only in well-differentiated tumors. **Conclusions.** Tumor differentiation, rather than inflammatory signaling, modulates CX3CR1 expression in PanINs and PDAC. CX3CR1 expression pattern suggests its early involvement in PDAC progression, outlining a potential target for interfering with the PanIN transition to invasive cancer.

INTRODUCTION

Pancreatic cancer, namely ductal adenocarcinoma (PDAC), is a major health problem, the death rate approaching the incidence of the disease (Hidalgo, 2010). The vast majority of PDACs are diagnosed at an advanced stage, beyond any possibility of cure. A better understanding of the early neoplastic changes in the pancreas might help anticipating the diagnosis and counteracting the rapid progression of pancreatic cancer. Microscopic pre-cancerous lesions (Pancreatic Intraepithelial Neoplasia, PanIN) house *KRAS* mutations (Jones *et al*, 2008; Sipos *et al*, 2009) and accumulate further gene damage along progression to cancer (Jones *et al*, 2008). However, gene damage is not the only driver of pancreatic carcinogenesis, PDAC being characterized by a dense stromal reaction, referred to as desmoplasia (Farrow *et al*, 2008), which is an active partner in disease progression (Hwang *et al*, 2008; Vonlaufen *et al*, 2008). Stromal cells produce cytokines and growth factors that establish an active tumour-stroma cross-talk (Farrow *et al*, 2008; Hwang *et al*, 2008; Vonlaufen *et al*, 2008). Mediators of the cross-talk between cancer and microenvironment include chemokines, small chemotactic cytokines, and their receptors (Balkwill, 2012; Mantovani *et al*, 2008; Wang *et al*, 2008). Tumour expression of chemotactic molecules modifies the behaviour of cancer cells and tumour progression. The gain of expression of chemokine receptors enhances cancer invasion and spread by mediating cancer cell trafficking and metastasis homing (Mantovani *et al*, 2010). Chemokine expression in cancer may also affect immune recognition by the host, and may promote autocrine loops favouring the survival of tumour cells (Balkwill, 2012; Wang *et al*, 2008). The expression of the chemokine receptor CXCR4 has been associated with an enhanced progression of PDAC, and with stemness of cells with metastatic potential (Hermann *et al*, 2007; Marchesi *et al*, 2004). Surprisingly, the expression of CXCR4 and of its ligand CXCL12 begins in the pre-invasive

stages of pancreatic neoplasia (Thomas *et al*, 2008). The relevance of chemokine receptors in PDAC spread, has been recently strengthened by the finding that tumour cells also express the chemokine receptor CX3CR1 (Marchesi *et al*, 2008). CX3CR1 forms a high-affinity axis (CX3CR1-CL1) with its unique ligand CX3CL1 (also referred to as Fractalkine/Neurotactin), a mucin-chemokine hybrid expressed by endothelial cells and neurons, either as a membrane-anchored adhesion molecule, or as a secreted chemoattractant (Bazan *et al*, 1997; Pan *et al*, 1997). CX3CR1 expression was associated with the degree of peri-neural invasion (PNI), and PDAC cells expressing CX3CR1 were found to disrupt peripheral nerves once xenografted in mice (Marchesi *et al*, 2008). It is unknown whether the expression of the receptor occurs already in pre-invasive stages of pancreatic carcinogenesis, or alternatively, only in invasive cancer. Furthermore, limited information is available as to the expression of the ligand in PDAC (Xu *et al*, 2012), and as to whether tumor features and microenvironment crosstalk may affect CX3CR1-CL1 status.

This study was aimed to investigate whether pancreatic cancer expresses CX3CR1-CL1, already gaining the expression of these chemotactic molecules in pre-invasive stages, and whether an association exists with PDAC features and outcome. To these aims, we assessed the expression of CX3CR1-CL1 in human PanINs and invasive cancers, as well as in PanINs of a *Kras*-mutated mouse model of pancreatic cancer, and tested their responsiveness to cytokines and their expression in tumors propagated *in vivo*.

MATERIALS AND METHODS

Patients and tissues specimens. We retrospectively retrieved the tissue specimens from 104 patients who underwent resective surgery for pancreatic cancer at the Humanitas Clinical and Research Center, Rozzano, Milan, Italy. Seventy-six PDAC also harbored precursor lesions.

For each patient included in the study, demographics and complete pathological data at diagnosis were available. Specimens were reviewed by pathologists unaware of molecular data. Tumour pathological staging, histopathological typing, tumour grade, and presence or absence of PNI and/or of extramural vein invasion, were assessed in each PDAC. The absence of nodal involvement was considered appropriate only for PDAC in which ≥ 12 lymph-nodes had been assessed. The study was conducted in accordance with the guidelines of the Ethics Committee of the Hospital, and the referring physician obtained the informed consent of the patients to the treatment of their personal data at the time of surgery. The overall survival (OS) was calculated from diagnosis until death, and deceases were documented by obtaining the death certificate in every case.

Mouse models of pancreatic cancer. Female and male mice carrying mutated *Kras*^{G12D} or *Trp53*^{R172H} (C57BL/6;129SvJae H-2b;d) under the endogenous promoter and flanked by Lox-STOP-Lox cassettes were bred by us (Hingorani *et al*, 2005). PdxCre/LSL-*Kras*^{G12D} and PdxCre/LSL-*Kras*^{G12D}-*Trp53*^{R172H} double mutant mice, developing PDAC, were generated by crossing them with C57BL/6 mice, expressing Cre recombinase, under a specific pancreatic transcriptional factor Pdx-1 (pancreatic duodenum homeobox 1) promoter. Mice were screened by PCR using tail DNA amplified by specific primers to Lox-P cassette flanking *K-ras* gene and Cre recombinase gene. Mice were kept under pathogen-free conditions at the Molecular Biology Center, University of Turin, and treated in accordance with European Union and University of Turin Guidelines. Pancreata, from wild type or PdxCre/LSL-*Kras*^{G12D} mutant

mice sacrificed at 1, 6 and 9 months from birth were subsequently fixed in formalin and embedded in paraffin.

Immunohistochemistry. Two μm -thick sections were processed for immunohistochemistry (IHC). After deparaffining and rehydration, the sections were immersed in antigen retrieval bath, incubated with 3% H_2O_2 for 15 min, and treated for two hours at room temperature with primary antibodies raised against CX3CR1 (Ab8021, polyclonal rabbit anti-human, Abcam, Cambridge, UK) and CX3CL1 (AF365, polyclonal goat anti-human, R&D Systems, Italy), or with rabbit or goat IgG (Dako, Milan, Italy) to serve as negative controls, followed by 30 minutes' incubation with the DAKO Envision system (Dako) or Anti-Goat Polymer kit (Biocare, S.Francisco, USA). Three-3'-diaminobenzidine tetrahydrochloride (Dako) was used as chromogen and nuclei were lightly counterstained with haematoxylin (Medit, Bergamo, Italy).

Following immunohistochemistry, in a subset of 15 PDAC harboring CX3CR1⁺ precursor lesions, PanINs were graded (54 PanIN-1, 43 PanIN-2, and 35 PanIN-3) and systematically evaluated for CX3CR1, CX3CL1, P16, and E-Cadherin expression according to the degree of dysplasia. P16/CDKN2A and E-Cadherin immunohistochemistry was performed with mouse monoclonal antibodies (Ab-7, 16P07, Neo Marker Lab Vision Westinghouse, CA, and clone NCH38, Dako, respectively).

In pancreata obtained from 5 PdxCre/LSL-*Kras*^{G12D} mutant mice, CX3CR1 immunohistochemistry was performed with the same antibody used for human tissues, CX3CL1 expression was investigated by a polyclonal rabbit anti-mouse antibody (Torrey Pines Biolabs, East Orange, NJ, USA), and recognition of S-100⁺ neural structures was achieved by monoclonal rabbit anti-mouse antibodies (Dako, Italy).

The intensity of CX3CR1 and CX3CL1 immunoreactivity was independently evaluated by two researchers (F.G. and C.G.). Immunoreactivity of infiltrating leucocytes and of endothelial cells served as positive internal controls for CX3CR1 and for CX3CL1, respectively. The semi-quantitative evaluation of immunoreactivity of neoplastic ductal cells was first attributed through a 0-to-3 score: 0 (absence of immunoreactivity), 1 (weak), 2 (moderate), and 3 (strong), according to the level of immunoreactivity of most ($\geq 70\%$) neoplastic cells (Marchesi *et al*, 2008). Samples with 0-1 and with 2-3 immunoreactivity were subsequently pooled together, in a binary classification.

CX3CR1 and CX3CL1 mRNA expression in human and mouse ductal cell-lines. Quantitative RT-PCR was employed to assess the expression of CX3CR1 and CX3CL1. Briefly, one μ l of sample cDNA was added to the PCR reaction mixture containing 2X Power SYBR Green Master Mix (Applied Biosystem, Life Technologies, Grand Island, NY, USA) and specific primers [for CX3CR1, 5'-GGGACTGTGTTTCCTGTCCAT, and CX3CR1 5'-GACACTCTTGGGCTTCTTGC-3'; for CX3CL1 5'-TGGCTGCTCCGCTTGGC-3', and CX3CL1, 5'-CCTGGTTCTGTTGATAGTGGA-TGAG-3'); 18s RNA was used as housekeeping gene. Data analysis was performed by comparative Delta Ct ($\Delta\Delta C_t$) method, and fold change calculated using $2^{-\Delta\Delta C_t}$.

Primary mouse pancreatic cell lines DT6606 (obtained from a tumour arisen in a PdxCre/LSL-*Kras*^{G12D} mouse) and K8484 (obtained from a tumour arisen in a PdxCre/LSL-*Kras*^{G12D}-*Trp53*^{R172H} mouse (Olive *et al*, 2009) and human pancreatic cancer cell line A8184, MiaPacaII and AspCI were maintained in RPMI with 10% fetal bovine serum (FBS), 100mg/ml of

penicillin G, 50mg/ml of Streptomycin and Ultraglutamine 2mM and growth at 37°C, 5% CO₂ incubator. Cells seeded at 10⁶ cells/mL in six-well plates were stimulated with: interleukin-1 beta (IL-1 β) (20ng/mL), tumor necrosis factor- α (TNF- α) (20 ng/mL) and interferon- γ (IFN- γ) (500 units/mL), interleukin-6 (IL-6) and transforming growth factor- β (TGF- β) (10ng/mL) (Peprotech, Rocky Hill, NJ) for 8 hours. Expression of *CX3CR1* and *CX3CL1* was assessed by qRT-PCR, as previous described by using primers for murine CX3CR1 5'-

ATTCTTCATCACCGTCATCAG, 5'-ACTAATGGTGACACCGTGCT and for murine CX3CL1 5'-GCTATCAGCTAAACCAGGAGTC, 5'-AGAAGCGTCTGTGCTGTGTC.

Three-dimensional cell culture, immunofluorescence, and cell transplantation in syngenic mice. DT6606, K8484, A8184 and AspCI cells were mixed with Cultrex (5X10⁴ cells/250 microliter) and plated in a 24-well plate. Cells were fixed overnight in 4% PFA, washed twice with PBS/- plus 0.2% Tween. Immunofluorescence was performed after blockade of endogenous non-specific antigens and permeabilization by PBS/- plus 10% normal goat serum, and 0.03% Triton. Cells were treated with primary antibodies raised against CX3CR1 (Ab8021, Abcam, Cambridge, UK) overnight at 4°C, washed, and incubated with secondary antibody 1:2000 Alexa-fluor-488 (Invitrogen) for 1 hour at room temperature. Subsequently cells were incubated with 1:50 dilution of Alexa-fluor-594 phalloidin (Invitrogen). Nuclei were counter-stained with DAPI (Invitrogen) 1:10.000. Imaging was obtained by confocal microscopy (Olympus). For RT-PCR analysis mRNA from spheroid in culture was extracted by Trizol (Applied Biosystem), according to manufacturer instruction, RT and analysis was performed as above described.

DT6606, K8484 and PANC02 cells were transplanted in syngenic C57BL/6 mice. We took advantage of a porous collagen biocompatible matrix, which provide a three dimensional spatial architecture scaffold for the cells (Collagen Sponge CS-35, KOKEN Co Ltd, Tokyo, Japan). Collagen Sponges were loaded with DT6606, K8484 and PANC02 cells ($10^6/1$ mL) and intra-dermal injected in 15, 8 and 5 C57BL/6 mice, respectively by a small incision on the left flank. Mice were sacrificed after 2 weeks and tumours were immediately resected and fixed in formalin.

Statistical analysis. Categorical associations between expression of CX3CR1 and CX3CL1 and clinical-pathological factors of PDAC were analyzed by Chi-square (or Fisher's test, when appropriate), while Student's T-test was employed for continuous variables.

Kaplan-Meier curves of overall survival were plotted. The log-rank test was used to compare the curves of patient subgroups. A Cox proportional hazards model was constructed to assess the role of fractalkine and its receptor in predicting the overall survival.

Analyses were done using Epi Info version 3.5.1 and STATISTICA 7.1. For each test, only two-sided p-values lower than 0.05 were considered statistically significant.

RESULTS

CX3CR1 expression in PDAC is associated with tumour glandular differentiation and perineural invasion. We previously showed that the chemokine receptor CX3CR1 is up regulated in pancreatic cancer compared to normal exocrine tissue, and that receptor expression was involved in tumor PNI (Bazan *et al*, 1997). In this study we investigated in an independent cohort whether the specific ligand CX3CL1 is also expressed in PDAC, and whether both molecules are up regulated already in pre-neoplastic lesions (PanINs).

In normal pancreas, CX3CR1 expression by immunohistochemistry was faint to negative in exocrine cells but positive in endocrine islets (Fig. 1A). Conversely, cancer cells in 56 of 104 (53.9%) PDAC expressed CX3CR1. In PDACs, CX3CR1 expression was associated with a well-to-moderate differentiation (65.1% of CX3CR1⁺ in G1/G2 PDAC vs 37.5% CX3CR1⁺ in G3 PDAC; $p=0.005$) (Fig. 1B-C), as well as with the presence of PNI (63.1% of CX3CR1⁺ neural-invasive PDAC vs 38.5% of CX3CR1⁺ non-neural-invasive PDAC, $p=0.01$) (Table 1), this latter being not associated with tumor grade. **Notably, in our series the presence of PNI was not associated with tumor differentiation (40 of 63, 63.5%, G1-G2 neuroinvasive cancers vs 24/40, 60%, G3 neuroinvasive ones, $p=0.44$).** In addition, by stratifying cancers according to their differentiation we found that CX3CR1⁺ differentiated tumors were more frequently neural invasive (30/40, 75%) than CX3CR1⁻ cancers (10/23, 43.5%; $p=0.008$), while in poorly-differentiated cases the frequency of PNI was similar in CX3CR1⁺ (10/15, 66%) and in CX3CR1⁻ cancers (14/25, 56%; $p=0.37$) (Fig. 1G).

In the tumour microenvironment, vessels and infiltrating inflammatory cells were also CX3CR1⁺ (Fig. 1B, inset).

In non-neoplastic pancreas, CX3CL1 was faintly expressed in exocrine cells and endocrine islets, and expressed by neural cell bodies (Fig. 1D-E) but not by neural processes.

Seventy of 104 (67.3%) PDAC showed immunoreactivity for CX3CL1 (Fig. 1F). No significant correlation was found between CX3CL1 expression and cancer features (Table 1), but the ligand was more frequently expressed in CX3CR1⁺ (45/56, 80.3%) than in CX3CR1⁻ (23/48, 51.1%; $p=0.002$) PDAC.

At multivariate analysis, better tumour differentiation ($p=0.008$), PNI ($p=0.03$), and ligand expression ($p=0.003$) were all independently associated with CX3CR1 expression (Table 2).

CX3CR1 expression in cancer is associated with better patient survival. We assessed patient survival according to CX3CR1 and CX3CL1 status in PDAC. Patients with CX3CR1⁺ PDAC had a longer survival (mean 22.6 months; 95% CI, 15.7-29.5 months) than patients with receptor-negative cancers (14.45 months; 95% CI, 11.0-17.8 months), although the difference was not statistically significant (log-rank test, $p=0.07$). However, considering only patients with radical surgery and no surgery-related mortality, those with CX3CR1⁺ PDAC had a significantly better survival (31.95 months; 95% CI, 22.7-41.2 months) than those with CX3CR1-immunonegative cancers (18.9 months; 95% CI, 14.9-22.9 months; log-rank test, $p=0.05$) (Fig. 1H). No survival difference was observed according to CX3CL1 status in PDAC ($P=0.95$). In this set of patients, the analysis by Cox proportional-hazard model showed that the tumor expression of CX3CR1 was associated with a better survival (H.R. 0.56, 95% C.I., 0.32-1.00; $p=0.05$), independently of tumor grade and PNI. Adjusting the model by tumor stage resulted in loss of statistical significance, although patients with CX3CR1⁺ PDACs retained tendency to better survival (H.R. 0.58, 95% C.I., 0.32-1.04; $p=0.07$).

Precursor lesions of pancreatic cancer express CX3CR1 and CX3CL1. We evaluated receptor and ligand status in PanINs, to assess whether their expression appears before an invasive stage. In our series, 76 (73.1%) PDAC specimens harbored precursor lesions.

Precursor lesions were in general more frequently positive for CX3CR1 than PDACs (61, 80.3%, vs 42, 55.3%, respectively; $p=0.002$), and specifically PanINs in specimens of undifferentiated G3 cancers were more frequently CX3CR1⁺ than PDAC (22/28, 78.6%, vs 10/28, 35.6%, respectively; $p=0.001$) (Figure 2A). CX3CL1 was also strongly up regulated in PanINs (40/48, 83.3%) (Figure 2A) and, as observed for the receptor, ligand expression was higher in PanINs (26/28, 92.9%) than in associated G3 PDACs (17/28, 60.7%; $p=0.005$) (Figure 2A).

Next, we studied ligand and receptor expression in precursor lesions according to their degree of dysplasia, characterized by morphological analysis and molecular phenotype (E-Cadherin and p16/CDKN2A; Supplementary Fig. S1). Of 15 PDAC specimens analyzed, 8/8 G1-G2 tumors were CX3CR1⁺, while 3/7 G3 tumors were CX3CR1⁺. In the set of graded PanINs, the frequency of CX3CR1⁺ precursor lesions increased from PanINs-1 to through PanINs-3 ($p< 0.001$; Figure 2B); conversely, CX3CL1 expression decreased from PanIN-1 through PanIN-2 and PanIN-3 ($p=0.003$ Figure 2B). Representative images of CX3CR1 and CX3CL1 immunohistochemical staining in human PanINs are shown in Fig. 3A.

Overall, up regulation of both CX3CR1 and CX3CL1 is an early feature of pancreatic carcinogenesis, being expressed already in PanINs-1. While positivity for both molecules is maintained through carcinoma stage if PDAC retains well-to-moderate differentiation, poorly differentiated tumors tend to lose their expression. These results indicate that the expression of the CX3CL1/CX3CR1 axis is a distinguishing feature of tumors with glandular morphology.

To confirm these findings, we studied precursor lesions in the PdxCre/LSL-*Kras*^{G12D} mouse model of pancreatic cancer. As in humans, the normal exocrine pancreas of mice sacrificed before the development of precursor lesions (<6 months) did not show CX3CR1 or

CX3CL1 expression. Differently, CX3CR1 and CX3CL1 were expressed in the PanINs of mice older than 6 months of age, irrespectively of the degree of dysplasia (Fig. 3B).

To evaluate whether precursor lesions can mimic PNI, we stained the pancreata of 5 PdxCre/LSL-*Kras*^{G12D} mice with anti-S-100. Immunostaining revealed a small fraction (13 of 400; 3.25%) of precursor lesions either in proximity of, or in contact with, neural structures (Fig. 3B).

CX3CR1 is un-responsive to inflammatory stimuli, but its expression is enhanced in spheroids *in vitro*, and in well-differentiated tumor glands *in vivo*. We tested the responsiveness of *CX3CR1* and *-CL1* expression to cytokines in pancreatic tumor cells. In DT6606 cells, derived from tumors arising in PdxCre/LSL-*Kras*^{G12D} mouse model, and in K8484 cells, derived from tumors of PdxCre/LSL-*Kras*^{G12D}-*Trp53*^{R172H} model, the only observed change of *CX3CR1* mRNA levels was a decrease following TNF- α plus IFN- γ treatment in DT6606 cells. Differently, TNF- α plus IFN- γ increased while TGF- β down-regulated the expression of *CX3CL1* mRNA in both murine tumor cell-lines, an effect obtained also by IL-1 β and IL-6 treatment in K8484 cells (Fig. 4).

In human PDAC cells (A8184, AspCI and MiaPacaII) inflammatory cytokines did not affect the mRNA levels of the receptor, but TNF- α plus IFN- γ and IL-1 β strongly increased the expression of the ligand. Like in cells derived from PdxCre/LSL-*Kras*^{G12D} and *-Kras*^{G12D}-*Trp53*^{R172H}, IL-6 and TGF- β stimulation down-regulated the ligand in A8184 and AspCI cells (Fig. 4).

The preferential expression of CX3CR1 in precursor lesions and differentiated PDAC, together with the lack of responsiveness to inflammatory signals, suggests that glandular differentiation may affect CX3CR1 status. Accordingly, we tested whether the formation of gland-like structures can induce CX3CR1 expression *in vitro*. DT6606 and K8484 cells

propagation as spheroids showed a spontaneous and significant *CX3CR1* mRNA and protein increase as compared to monolayer cell culture, in which less than 5% and 20% of cells, respectively, were *CX3CR1*⁺. Immunofluorescence revealed a uniform *CX3CR1* expression in the cells positioned in the outer portion of the glandular structure formed by DT6606 and K8484 spheroids. The same immune-positivity was detectable in human cancer AspCI cell spheroids, while in A8184 cells, receptor expression was not significantly increased in spheroids with respect to monolayers (Fig. 5A-D).

To confirm the association between glandular differentiation and *CX3CR1* expression, we grafted pancreatic tumour cells in syngenic C57BL/6 mice. Sub-cutaneous injection of DT6606 cells in 15 mice and of K8484 cells in 8 mice ensued in tumor growth in 11 (73.3%) and in 8 (100%) animals, respectively. The resulting tumors were well differentiated and showed intense and homogeneous *CX3CR1* immunostaining. Differently, PANC02 cells efficiently propagated as un-differentiated cancers in 5/5 mice, but none of these cancers expressed the receptor (Fig. 5E).

DISCUSSION

Our extensive studies in human pancreatic cancer and precursor lesions, in a genetic mouse model, as well as *in vitro* and *in vivo*, elucidate important aspects of *CX3CR1*-*CL1* expression in pancreatic carcinogenesis. First, we found that human PanINs and well-differentiated PDACs express *CX3CR1* more frequently than poorly differentiated cancers. Furthermore, *CX3CR1* expression was significantly associated with PNI only in differentiated PDAC. The presence of the receptor already in pre-invasive precursor lesions indicates an early timing of expression in pancreatic carcinogenesis. Such an early appearance was experimentally mirrored by the results in the animal model. Next, the presence of the receptor in differentiated

cancers would equip soon these tumors with the capability to follow a chemotactic drive, contributing to PNI (Marchesi *et al*, 2008). On the other side, our findings imply that poorly differentiated PDACs do not take advantage of CX3CL1-driven chemotaxis and adhesion in their spread. Such a difference in the expression of chemotactic receptors depending upon tumor differentiation is not restricted to CX3CR1, as CXCR7 was conversely found to be more frequently expressed in poorly differentiated pancreatic cancers (Gebauer *et al*, 2011). Accordingly, tumor grade, an important determinant in PDAC outcome (Helm *et al*, 2009; Luttges *et al*, 2000), accounts for the differential expression of chemokine receptors. As the precursor lesions in the context of poorly differentiated PDAC expressed CX3CR1, we can infer that cancer cells no longer express this receptor when clonal selection privileges the outgrowth of undifferentiated clones. It is thus conceivable that the sensitivity of pancreatic cancer to chemokine attraction varies along tumor evolution (Buckle *et al*, 2012; Kim *et al*, 2005), such as changes in tumor differentiation.

The pro-invasive role of CX3CR1, which is foreseeable after the transition from PanIN to invasive cancer, remains otherwise elusive in pre-invasive lesions (Balkwill, 2012). In a mouse model of pancreatic cancer, PanIN cells can cross the basement membrane and delaminate in the microenvironment before any invasive behavior can be detected by histological criteria, indicating that PanINs may already harbor cells with invasive features (Rhim *et al*, 2012). Such invasive and tumor initiating cells from PanINs lacked E-Cadherin (Rhim *et al*, 2012), and in keeping with this notion we found a marked decrease of E-Cadherin expression in CX3CR1+ PanINs. Additional studies should assess whether delaminating cells from PanINs express CX3CR1, anticipating the involvement of chemotaxis in PNI.

We have previously reported the association between CX3CR1 expression, marked PNI, and shorter progression-free survival (Marchesi *et al*, 2008). In this independent cohort, we

found that patients with CX3CR1⁺ PDAC had a longer overall survival. PNI is a predictor of tumor recurrence, but tumor grade is the only histological feature of pancreatic cancer associated with worse survival (Helm *et al*, 2009; Shimada *et al*, 2011) . Consistently, tumor grade, but not PNI, was a predictor of patient survival in our series, and the tumor expression of CX3CR1 retained prognostic value once adjusted for tumor grade and stage in a Cox model. Thus, the association between CX3CR1 tumor expression and better patient survival may simply reflect an earlier disease. Differently, Xu and coll. reported that CX3CR1 status in PDAC has no prognostic value, while CX3CL1 expression would predict a worse prognosis (Xu *et al*, 2012). In this series, however, radical surgery was not considered, and average survival was almost half than in our, implying that patients with rather advanced disease have been studied. Considering that the invasive profile drawn by chemokine receptors changes with tumor evolution (Kim *et al*, 2005; Ottaviano *et al*, 2006), our results suggest that CX3CR1 could be foreseen as a biomarker paralleling early invasiveness, a feature that innovative imaging techniques might help to address (Buckle *et al*, 2012). If this were the case, the axis CX3-CR1-CL1 would be a candidate target for interfering with invasion at the transition to invasive cancer.

In the second instance, our experimental results provide support to the associative data. CX3CR1 was unresponsive to inflammatory signaling *in vitro*, but was modulated by the structural organization of tumor cells, both *in vitro* and *in vivo*. In parallel with findings in human PDAC, mouse tumor cells DT6606 cells expressed CX3CR1 *in vitro* only if cultured as spheroids, as they did *in vivo* growing as gland-forming tumours. Conversely, growth of undifferentiated PANC02 cells resulted in CX3CR1-negative cancers *in vivo*. Thus, experimental models confirming that tumor differentiation is crucial in setting CX3CR1 expression in tumor cell, reveal that the three-dimensional organization of neoplastic cells is a

crucial factor for the expression of a chemotactic receptor. Although we did not investigate the signaling coupling the organization of tumor cells with CX3CR1 expression, our data underline that the expression of the receptor is unlikely dependent upon micro-environmental cross-talk, rather being determined by tumor morphological features. Differently, CX3CL1 expression was strongly driven by inflammation *in vitro*. Pro-inflammatory TNF- α plus IFN- γ induced a higher increase of *CX3CL1* mRNA in *Kras*-mutated than in isogenic but *p53*-mutated ductal cells, confirming that *p53* damage dampens *CX3CL1* responsiveness along tumor progression (Algul *et al*, 2007; Jones *et al*, 2008; Shiraishi *et al*, 2000; Sipos *et al*, 2009). These results help to explain for the reduced rate of CX3CL1 expression in PanINs with increasing dysplasia, known to be coupled with *P53* damage (Remmers *et al*, 2011). The high rate of CX3CL1 expression in PanINs-1 is consistent with other data showing that the chemotactic recruiters of leukocytes appear with the first intraepithelial stages of human pancreatic carcinogenesis, decreasing thereafter (Hiraoka *et al*). The responsiveness of *CX3CL1* to cytokines observed in pancreatic tumor cells also fits with its involvement in the tumour-stroma cross-talk contributing to the desmoplastic reaction (Hiraoka *et al*), as shown in chronic pancreatitis (Ceyhan *et al*, 2009). Joined with previous findings (Thomas *et al*, 2008), our results firmly indicate that the switch-on of chemotactic molecules is a common and early feature of pancreatic carcinogenesis, encouraging the development of strategies aimed to interfere with the CX3-CR1-CL1 axis to constrain early on the invasiveness of differentiated pancreatic cancer.

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Titles and legends to figures

Figure 1. CX3CR1 and CX3CL1 expression in normal pancreas and in pancreatic cancer. A, CX3CR1 expression in normal pancreas, B in well- and C in poorly-differentiated PDAC, and (B inset) in inflammatory cells in tumour microenvironment. D, CX3CL1 expression in normal pancreas, E in neural cell bodies, and F in PDAC (box, 40x magnification of a CX3CL1+ PDAC). Objective magnification 10x (A,B, C, D, and F); panel E and inset in panel B, 40x. G, Frequency of CX3CR1⁺ and CX3CR1⁻ PDAC with PNI according to tumor differentiation (Grade, G1-G2 well to moderately differentiated PDAC; G3, poorly differentiated PDAC). H, Kaplan-Meier curves for overall survival of patients after radical resection of PDAC (n=67), according to the status of CX3-CR1 (left panel) and -CL1 (right panel) in cancer. P-values by log-rank test.

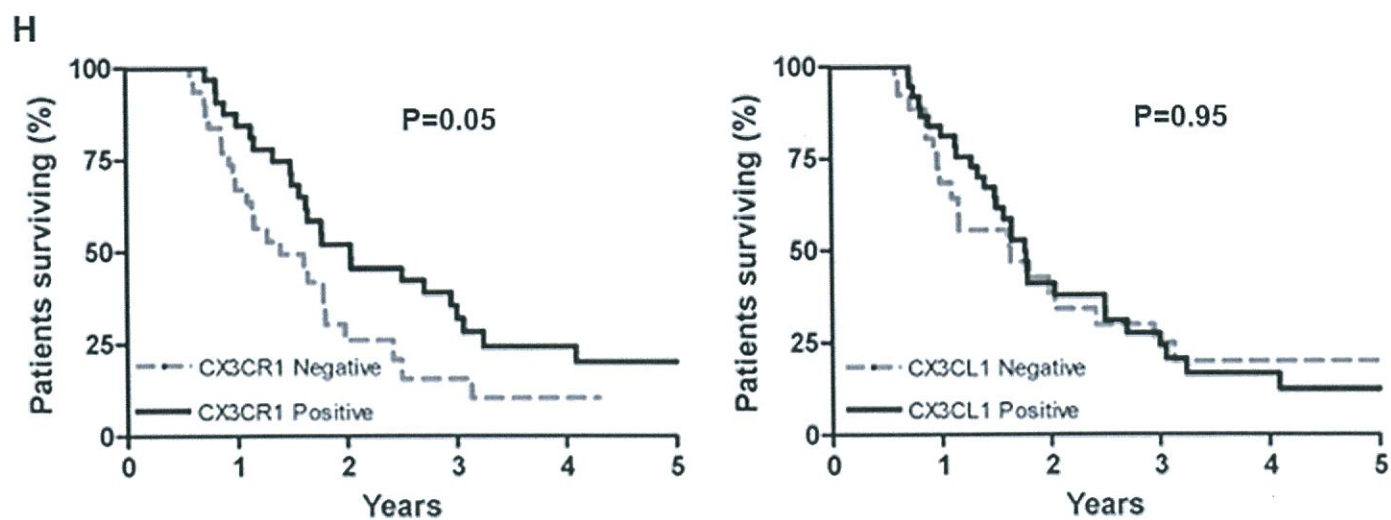
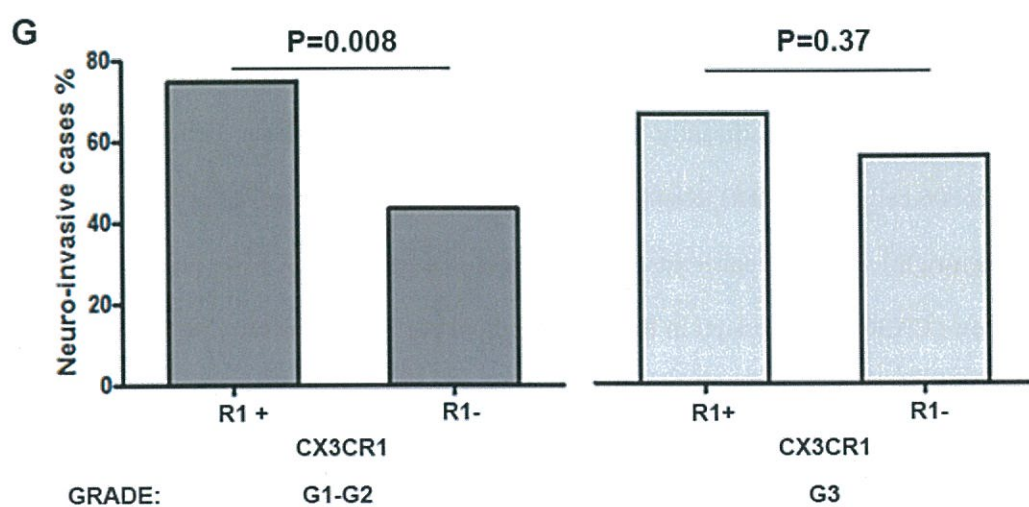
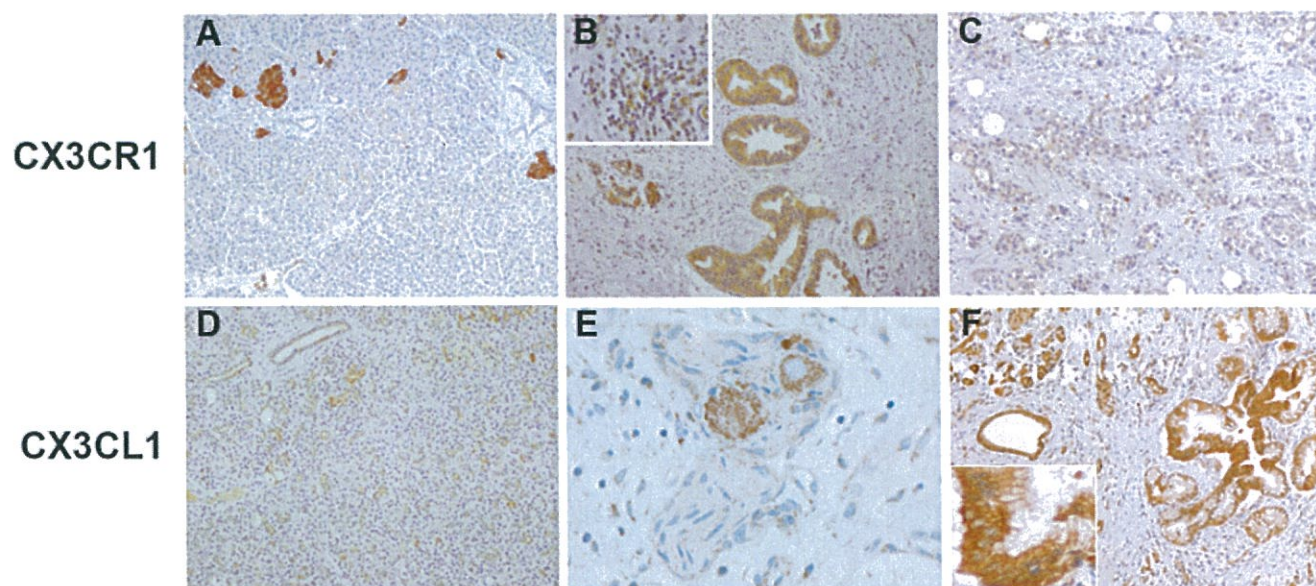
Figure 2. Expression of CX3CR1 and CX3CL1 in precursor lesions according to tumor differentiation and PanIN degree. (A) Comparison of the expression rate of CX3CR1 (upper panels) and CX3CL1 (lower panels) in paired precursor lesions and invasive cancers within the same tissue specimens (n=76), according to the degree of pancreatic cancer differentiation. Number within bars, number of specimens. (B) Rates of CX3CR1⁺ (upper panels) and CX3CL1⁺ (lower panels) PanINs according their degree of dysplasia. Numbers within bars, number of PanINs. G1-G2 tumors, left panels; G3 tumors, right panels; p-values by Fisher exact test.

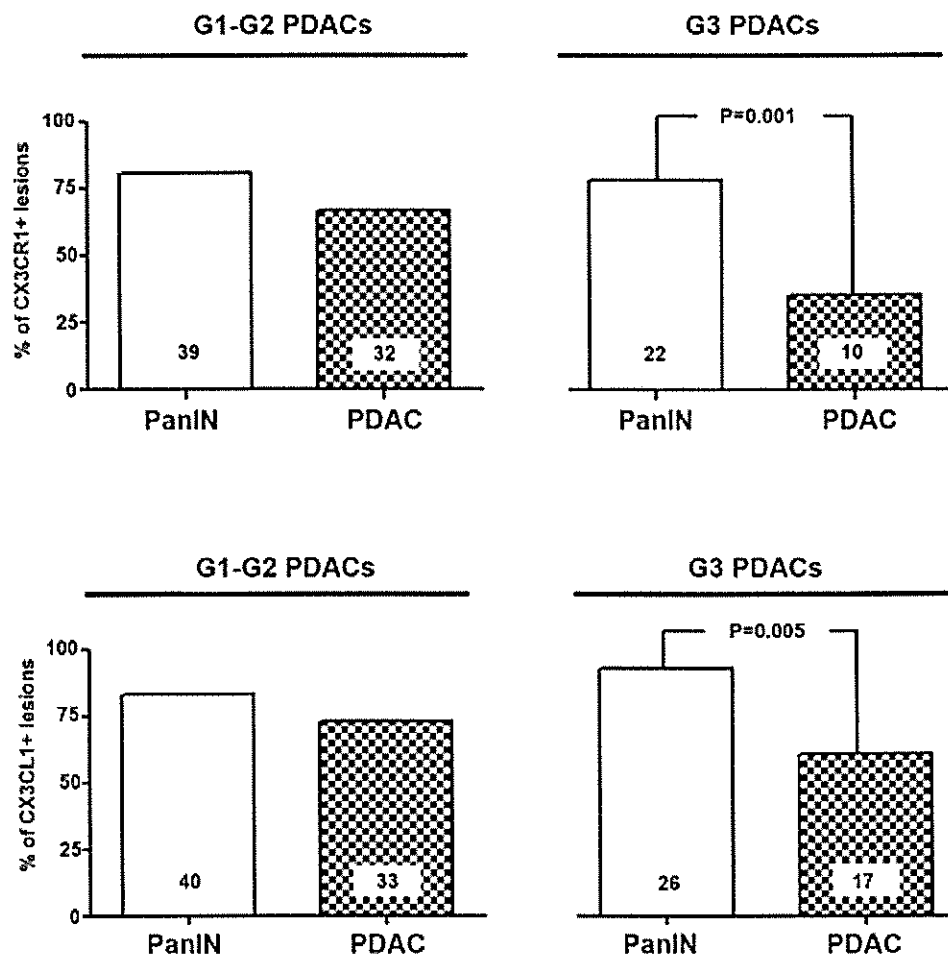
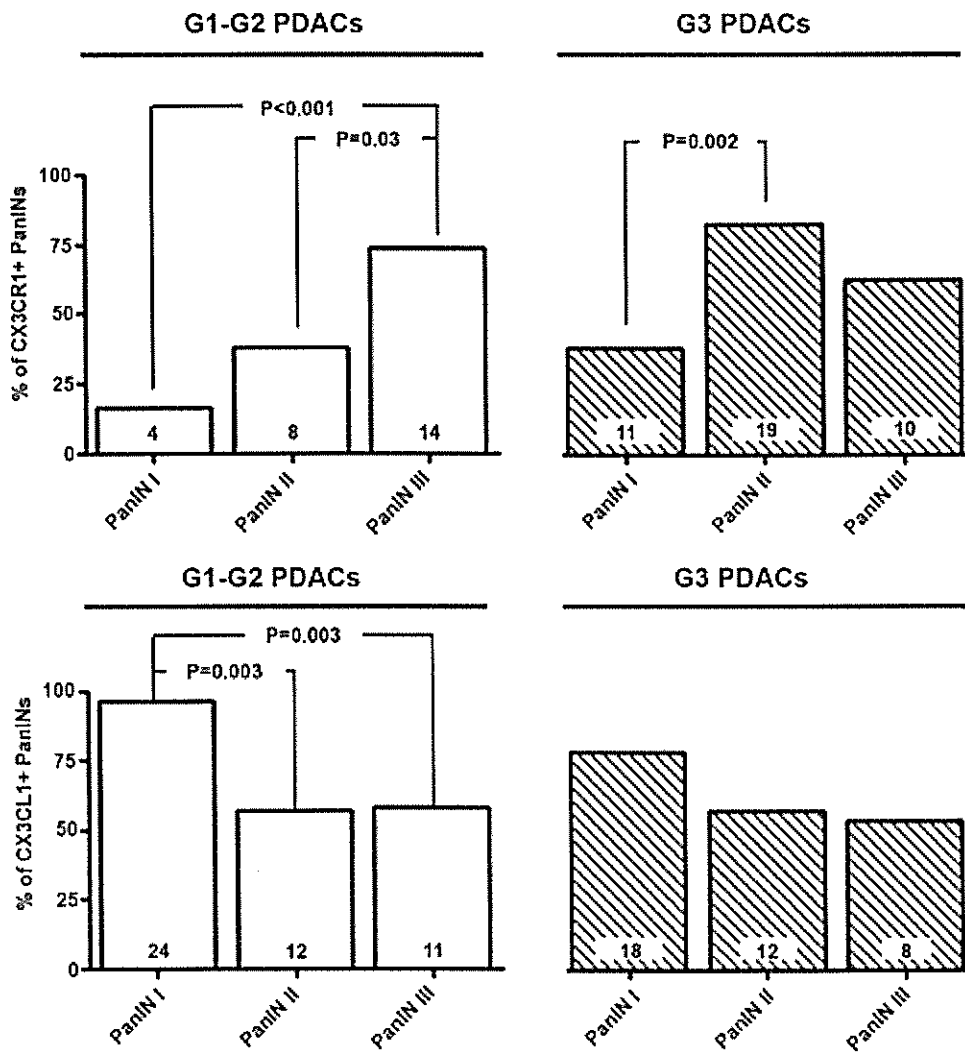
Figure 3. CX3CR1 and CX3CL1 expression in precursor lesions of pancreatic cancer. A, Left panels, human PanINs; right panels, PanINs of PdxCre/LSL-Kras^{G12D} mice. Objective magnification, 20x (human PanINs), and 40X (mice PanINs). B, precursor lesions adjacent to neural structures. Detection of precursor lesions close to (left panel) or in tight contact with

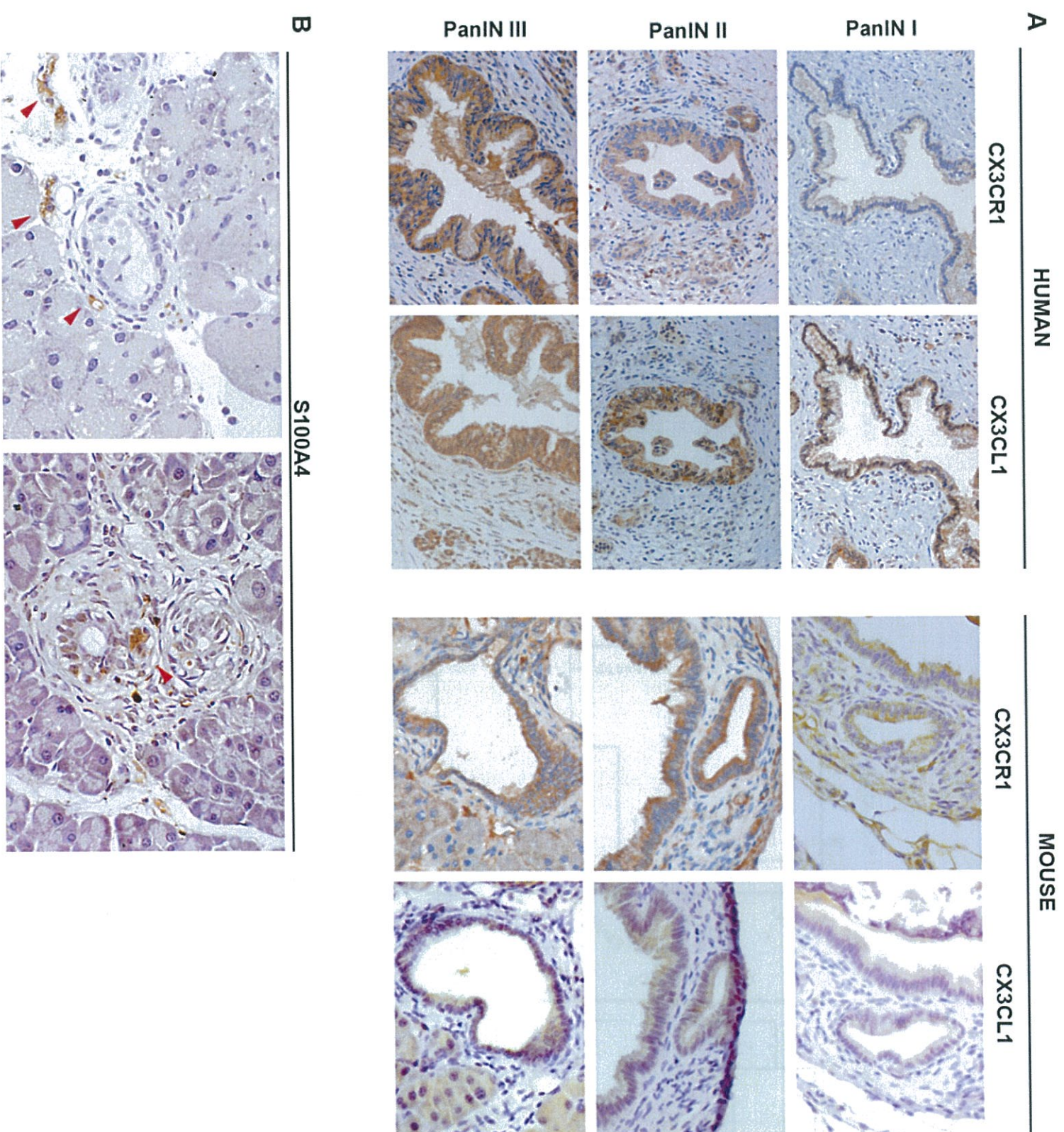
(right panel) S-100⁺ neural structures (red arrows) in pancreata of 6 month old *PdxCre/LSL-Kras^{G12D}* mice. Magnification, 20X.

Figure 4. Effects of microenvironmental stimuli on CX3CR1 and CX3CL1 expression in pancreatic tumoral cells. *CX3CR1* (left panels), and *CX3CL1* (right panels) mRNA fold-changes induced by stimulation with TNF- α +IFN γ , IL-1 β , IL-6, and by TGF- β in mouse DT6606 (*PdxCre/LSL-Kras^{G12}* model) and K8484 (*PdxCre/LSL-Kras^{G12D}-Trp53^{R172H}* model) cells, and in human A8184, *AspCI* and *MiaPacaII* pancreatic cancer cells.

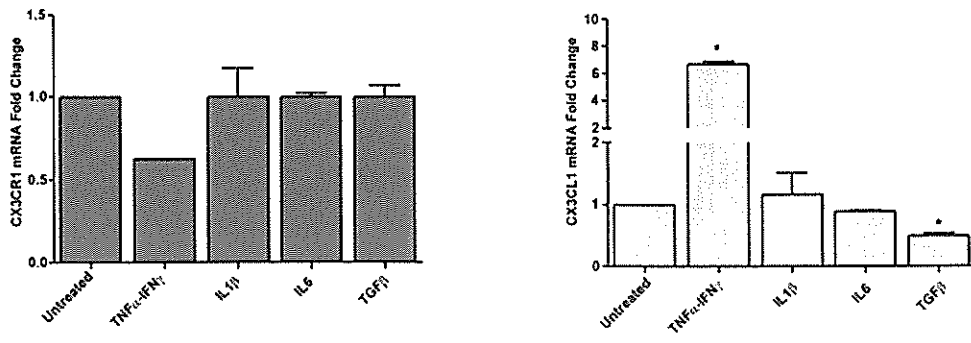
Figure 5. Effects on CX3CR1 expression of the three-dimensional organization of pancreatic tumor cells *in vitro*, and of tumor differentiation *in vivo*. A, spheroids formed by DT6606, K8484, *AspCI* and A8184, cells grown in Cultrex 3D[®] bulk. Objective magnification 40x. B, *CX3CR1* mRNA levels were significantly enhanced as compared to planar cell culture conditions in DT6606 cells (upper-left), K8484 (upper-right) and *AspCI* (down-left) but not in A8184 cells (down-right), grown as spheroids. C, immunofluorescence for CX3CR1 performed on spheroids, shows CX3CR1 expression in the outer spheroid portion in DT6606, K8484 and *AspCI* but not in A8184 cells (stained with phalloidin; scale bar, 10 μ m). D, cyto-fluorimetric analysis for CX3CR1 of PANC02, DT6606 and K8484 cell monolayers. E, Graph summarizes the rate of tumors growth in C57BL/6 mice and the corresponding expression status of CX3CR1 according to the injected cell-line. F, CX3CR1 was expressed by DT6606 and K8484 cells grafted in syngenic C57BL/6 mice and growing as differentiated tumour glands, but not by PANC02 cells growing as un-differentiated cancer (black and red arrows, tumour and inflammatory infiltrating cells, respectively).



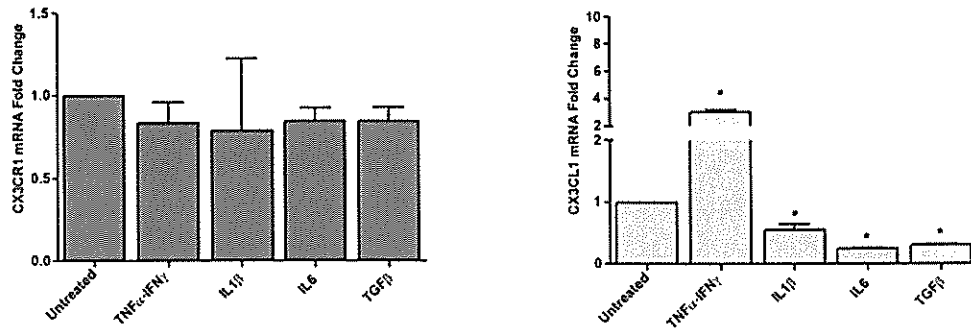
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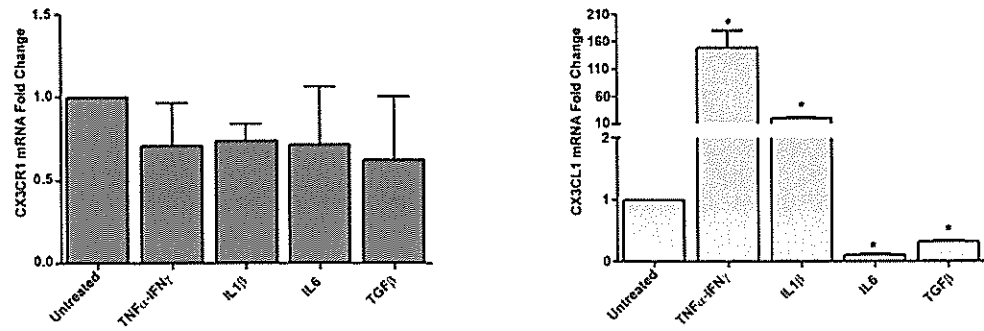
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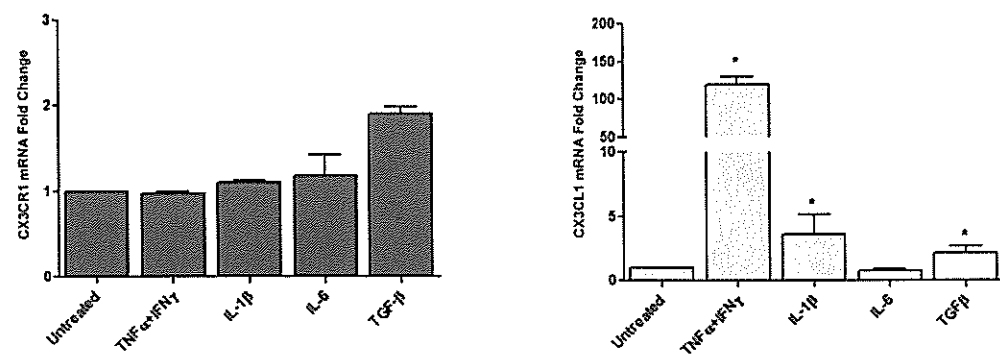
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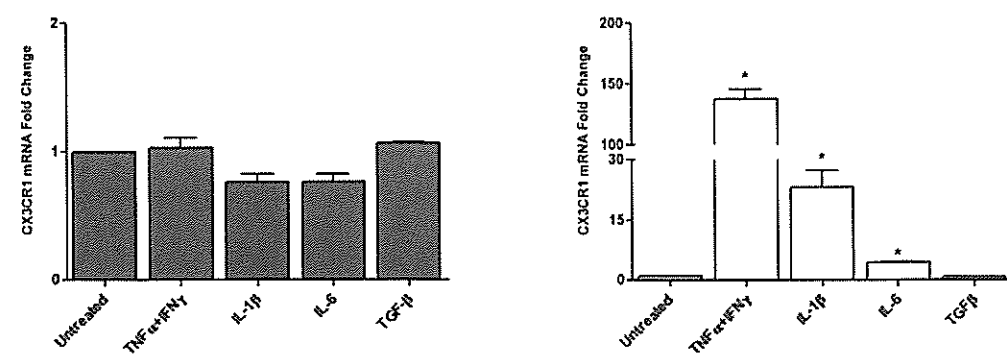
A8184



ASPCI



MiaPaca II



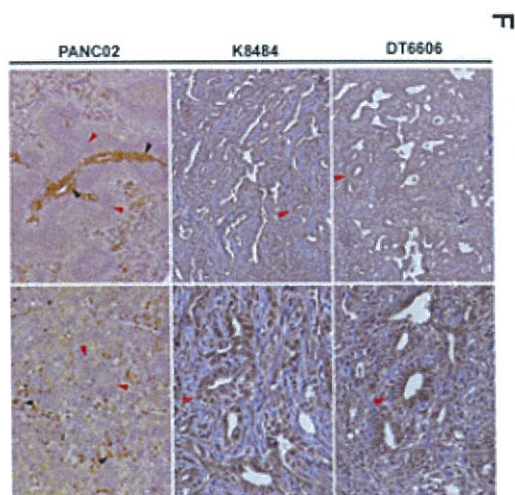
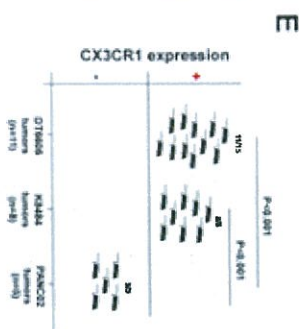
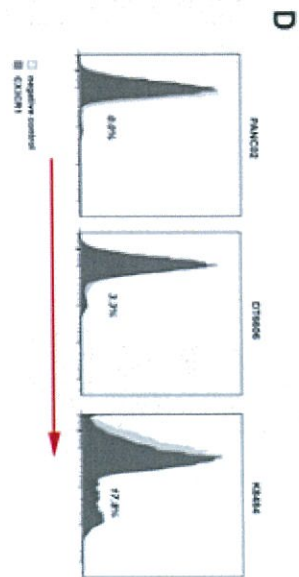
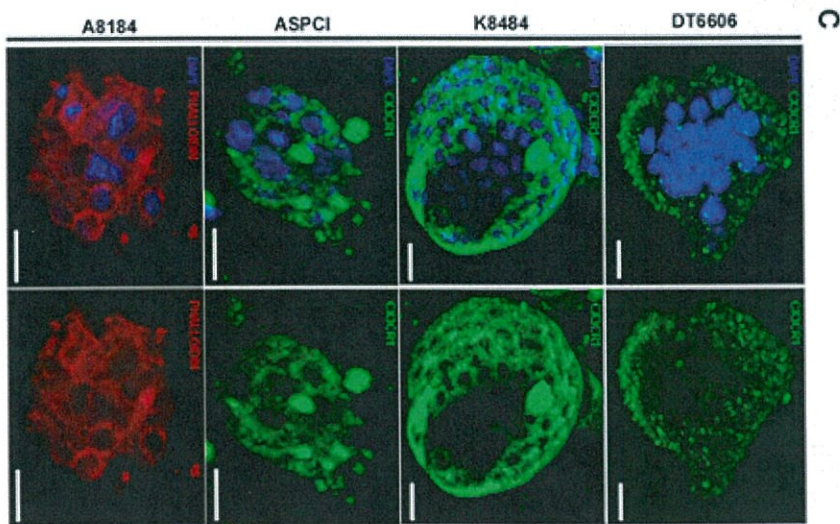
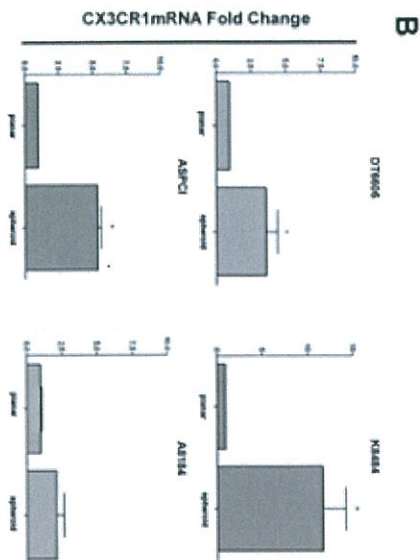
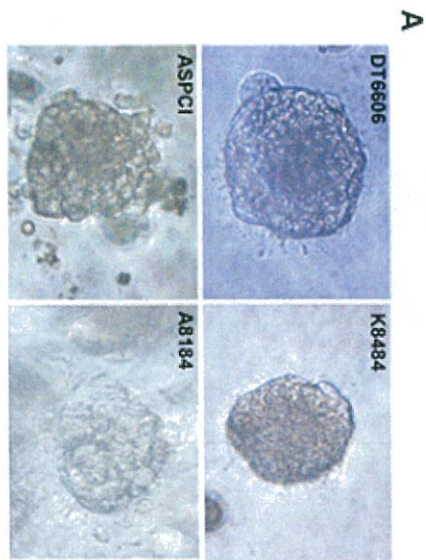


Table 1. CX3CR1 and CX3CL1 expression in 104 PDAC according to patients' demographics and to tumor features.

| | | Expression in PDAC | | | | | |
|---|-----------|----------------------------------|----------------------------------|-------------------|----------------------------------|----------------------------------|-------------------|
| | | CX3CR1 | | | CX3CL1 | | |
| | | Negative <i>n</i> =48 (46.1%) | Positive <i>n</i> =56 (53.9%) | P* | Negative <i>n</i> =34 (32.7%) | Positive <i>n</i> =70 (67.3%) | P* |
| Patient Age (years, <i>mean ± s.d.</i>) | | 65.6±9.4 | 63.9±7.6 | 0.29 | 63.4±9.5 | 65.3±7.9 | 0.28 |
| Patient Gender | male | 30 (47.6) | 32 (52.4) | 0.36 | 20 (31.7) | 43 (68.3) | 0.48 |
| | female | 18 (43.9) | 23 (56.1) | | 14 (34.1) | 27 (65.9) | |
| Tumor Location | head | 40 (47.1) | 45 (52.9) | 1.00 | 29 (34.1) | 56 (65.9) | 0.51 |
| | body-tail | 9 (47.4) | 10 (52.6) | | 5 (26.3) | 14 (73.7) | |
| Tumor Invasion | pT1-T2 | 4 (40.0) | 6 (60.0) | 0.55 | 4 (40.0) | 6 (60.0) | 0.64 |
| | pT3 | 41 (45.6) | 49 (54.4) | | 28 (31.1) | 62 (68.9) | |
| | pT4 | 3 (75.0) | 1 (25.0) | | 2 (50.0) | 2 (50.0) | |
| Nodal Metastasis | N0 | 3 (25.0) | 9 (75.0) | 0.29 [†] | 3 (25.0) | 9 (75.0) | 0.22 [‡] |
| | N+ | 35 (50.7) | 34 (49.3) | | 25 (36.2) | 44 (63.8) | |
| | Nx | 10 (43.5) | 13 (56.5) | | 6 (26.0) | 17 (64.0) | |
| Tumor Grade[#] | G1-G2 | 23 (36.5) | 40 (63.5) | 0.005 | 21 (33.3) | 42 (66.7) | 0.47 |
| | G3 | 25 (62.5) | 15 (37.5) | | 13 (32.5) | 27 (67.5) | |
| Tumor Diameter (cm, <i>mean ± s.d.</i>) | | 3.6±2.1 | 3.7±1.5 | 0.94 | 3.5±1.9 | 3.7±1.7 | 0.66 |
| Neural Invasion | no | 24 (61.5) | 15 (38.5) | 0.01 | 15 (31.2) | 24 (68.8) | 0.39 |
| | yes | 24 (36.9) | 41 (63.1) | | 19 (29.3) | 46 (70.7) | |
| Vascular Invasion | no | 23 (43.4) | 30 (56.6) | 0.28 | 18 (34.0) | 35 (66.0) | 0.39 |
| | yes | 25 (49.0) | 26 (51.0) | | 16 (31.4) | 35 (68.6) | |

*By CHI-square or Fisher's exact test as appropriate, except for patients' age and tumor diameter comparisons, for which Student's test was used.

^{†,‡} P-values of frequency distribution assessed in 81 (75.9%) PDAC with reliable nodal staging.

[#]Not determined in one PDAC, treated with neo-adjuvant radio-chemio-therapy.

Table 2. Tumor differentiation, peri-neural invasion, and ligand expression are independently associated with CX3CR1 expression.

| | CX3CR1 expression | | |
|-------------------|-------------------|------------|-------|
| | O.R. | 95%C.I. | P* |
| Tumor grade | | | |
| G1-G2 | 1.0 Ref. | | |
| G3 | 0.30 | 0.12-0.73 | 0.008 |
| Neural invasion | | | |
| No | 1.0 Ref. | | |
| Yes | 2.63 | 1.08-6.39 | 0.03 |
| CX3CL1 expression | | | |
| Negative | 1.0 Ref. | | |
| Positive | 3.99 | 1.57-10.14 | 0.003 |

*By multivariate logistic regression analysis